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the Leech *Helobdella* and the Origin of the Bilaterian Body Plan

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The *orthodenticle* orthologue *Lox22-Otx* was isolated from an annelid worm, the leech *Helobdella triserialis*. *In situ* hybridization reveals that embryonic expression of *Lox22-Otx* RNA is primarily restricted to an unsegmented head domain, including tissues in the foregut, surface ectoderm, and the head ganglion of the central nervous system. The patterns of head expression form concentric rings about the stomadeum and mark tissue domains that exhibit discrete behaviors during later morphogenesis and differentiation. Expression was also observed in one to two bilateral pairs of neurons in each segmental ganglion or neuromere of the body trunk. The largely head-specific expression of *Lox22-Otx* in this annelid species supports data from two other bilaterian phyla in suggesting the existence of a genetically defined head/trunk distinction. We suggest here that this head/trunk distinction is a synapomorphy of the Bilateria as a whole, and that it reflects the body plan of an early bilaterian ancestor. In addition, we discuss the possibility that the radial organization of gene expression and cell lineages in the leech's head domain may reflect the symmetry properties of a prebilaterian ancestor that had a radially symmetric body plan.

INTRODUCTION

The body plan of bilaterian animals is defined by a median plane of symmetry, and relative to that plane there is an organized differentiation of tissues along both the anteroposterior (AP) and dorsoventral (DV) axes. Pattern formation along the AP axis depends in large part on the differential expression of certain key regulatory genes. For example, genes of the Hox cluster have been found in all bilaterian animals examined, and their differential expression along the AP axis is a widely conserved mechanism for regional differentiation (McGinnis and Krumlauf, 1992; Salser *et al.*, 1993; Kourakis *et al.*, 1997).

However, some bilaterian animals use a different set of regulatory genes to pattern tissues at the extreme anterior end of their body plan. In *Drosophila*, for instance, the Pair Rule genes serve to define the 14 posterior parasegments of the body trunk, and the Hox (or homeotic) genes are required to specify the unique identities of those trunk parasegments (Lawrence, 1992). But the Pair Rule and Hox genes play little or no role in the embryonic patterning of the four yet more anterior head segments. Instead, this head domain is patterned by the nested expression of several other zygotic genes—such as *orthodenticle (otd)*—which have no role in trunk segmentation (Finkelstein and Perrimon, 1991; Cohen and Jürgens, 1991). A similar pattern of *otd* expression has also been found in the beetle *Tribolium* (Li *et al.*, 1996). Thus, the AP axis of the insect embryo can be divided into head and trunk domains whose development relies upon distinct regulatory gene networks.

The head genes that were originally identified in *Drosophila* have an outwardly similar pattern of regional utilization in the chordates, another phylogenetically disparate bilaterian taxon. Orthologues of *otd* (called Otx genes) have been described in a variety of vertebrate chordates, and both their expression (Boncinelli *et al.*, 1993; Li *et al.*, 1994; Pannese *et al.*, 1995) and function (Acampora *et al.*, 1995, 1996) are restricted to extreme anterior structures. The same is also true of the *Drosophila* gene *empty spiracles*

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(ems) (Finkelstein and Perrimon, 1991) and its vertebrate orthologues, the Emx genes (Boncinelli *et al.*, 1993; Yoshida *et al.*, 1997). For example, vertebrates express both Otx and Emx genes in the developing forebrain and midbrain, whereas the Hox genes only function more posteriorly in segments innervated by the hindbrain and spinal cord (McGinnis and Krumlauf, 1992). Cephalochordates and larval urochordates also express their Otx genes specifically in extreme anterior structures (Williams and Holland, 1996; Wada *et al.*, 1996), even though the tissue organization of their neuraxis is quite distinct from that of vertebrates. It has been suggested that these similarities in overall AP deployment of head gene and Hox gene expression domains in chordates and insects are a shared ancestral character or symplesiomorphy, and if this interpretation is correct it would mean that the last common ancestor already possessed genetically distinct body regions homologous to the head and trunk domains of modern Bilateria (Holland, 1992; Arendt and Nübler-Jung, 1996).

This hypothesis has not yet received general acceptance, and data on Otx genes in a third bilaterian phylum, the echinoderms, have suggested a high degree of evolutionary lability in their utilization during development (Gan *et al.*, 1995; Lowe and Wray, 1997). Echinoderms are defined as Bilateria by their ancestry, but the bilateral symmetry expressed during embryonic and larval stages is replaced by pentaradial symmetry in the adult, which has no clear-cut AP axis nor anatomically distinguishable head and trunk regions. It is generally held that pentaradial symmetry is a synapomorphy (shared derived character) of the echinoderms, and arose by modification of a more typical bilaterian ancestor (Brusca and Brusca, 1990; Raff, 1996). Thus, it may be that the variability in Otx expression observed among echinoderms (Lowe and Wray, 1997) is a byproduct of their modified symmetry properties. Hence, additional bilaterian phyla must be examined to ascertain whether the genetic head/trunk distinction found in insects and chordates is widespread among the Bilateria as a whole.

To provide further insight into this question, we here describe the isolation and characterization of an Otx gene—designated *Lox22-Otx*—from a member of another bilaterian phylum, the glossiphoniid leech *Helobdella triserialis* (Annelida). Several lines of evidence suggest that leeches might also have a set of developmental regulatory genes that are specific to the head. The leech body plan is subdivided into a segmented trunk generated by embryonic stem cells called teloblasts, and an unsegmented head domain or prostomium whose ectodermal tissues arise independently from the embryonic micromeres (Shankland and Savage, 1997). All 32 trunk segments express a leech orthologue, *ht-en*, of the *engrailed (en)* gene (Wedeen and Weisblat, 1991), and also the anteriormost Hox gene, the *labial (lab)* orthologue *Lox7* (Kourakis *et al.*, 1997). But neither ht-en protein nor any of the known Hox gene products have been detected in the prostomial head tissues.

In this paper we show that the prostomial head domain is the primary site of *Lox22-Otx* expression in the *Helobdella* embryo. This finding strongly supports the idea that multiple bilaterian phyla share a genetically defined head domain at the anterior end of the AP axis, which implies in turn that a genetic distinction between head and trunk is very likely to have been a primitive feature of the bilaterian body plan. In addition, we report here that the expression pattern of *Lox22-Otx* within the leech's head domain is radially organized about its mouth, and propose an evolutionary model in which the radially organized features of the head domain reflect the radial symmetry of a prebilaterian ancestor.

MATERIALS AND METHODS

Leech Embryos

H. triserialis embryos were obtained from a laboratory breeding colony. The colony is maintained at room temperature in 1% artificial seawater and fed physid pond snails (Weisblat *et al.*, 1980). Embryos were raised in buffered saline medium (Torrence and Stuart, 1986) and the staging system and cell nomenclature were used as described by Stent *et al.*, (1992).

Polymerase Chain Reaction (PCR)

A portion of the *Lox22-Otx* homeobox was initially isolated by PCR amplification of cDNA from stage 9 embryos. Total RNA was isolated with RNAzol (Tel-Test, Inc.), and the Superscript Kit (Gibco, BRL) was used to generate first-strand cDNA. cDNA (500 ng) was diluted in a 50-μl volume containing 200 μM of each dNTP, 1× PCR optimizer kit buffer A (Invitrogen), 1 μg each primer and 2.5 units *Taq* polymerase (Boehringer-Mannheim). Reaction protocol was 5 min at 95°C, followed by 30 cycles of 30 s each at 94, 45, and 72°C. After 30 cycles, 1 μl of reaction mixture was removed and used as the template for 30 additional rounds of amplification under the same conditions.

Amplification of the *Lox22-Otx* homeobox was accomplished with a pair of degenerate oligonucleotide primers based upon highly conserved regions of the Otx genes: upstream primer Otx C [5′-A(C/T)CC(A/C/T/G)GA(C/T)AT(A/C/T)TT(C/T)ATG-3′] and downstream primer Otx B [5′-(A/C/T/G)C(G/T)(A/C/T/G)C(G/T)(A/G)TT(C/T)TT(A/G)AACC-3′]. Successful amplification was expected to yielded a fragment of 86 bp length corresponding to homeodomain codons 25–53. Primers were obtained from Amitof (Allston, MA) and GIBCO-BRL.

PCR products were separated on an 8% polyacrylamide gel, and a band of the expected size was excised, purified, and cloned into Bluescript KS^+ (Stratagene). Recombinant plasmids were transformed into XL1-Blue using standard procedures (Ausubel *et al.*, 1992). Individual clones were characterized by dideoxy sequencing (Sequenase kit, U.S. Biochemicals). One of the 15 clones sequenced encoded a homeodomain nearly identical to that of the *Drosophila* otd protein (see Results). The gene containing this otd-like sequence was designated *Lox22-Otx*.

Library Screening

The cloned PCR fragment of *Lox22-Otx* was used to screen 8×10^4 recombinant clones of an *H. triserialis* genomic DNA library in bacteriophage EMBL3 (Wedeen *et al.*, 1990). Hybrid-

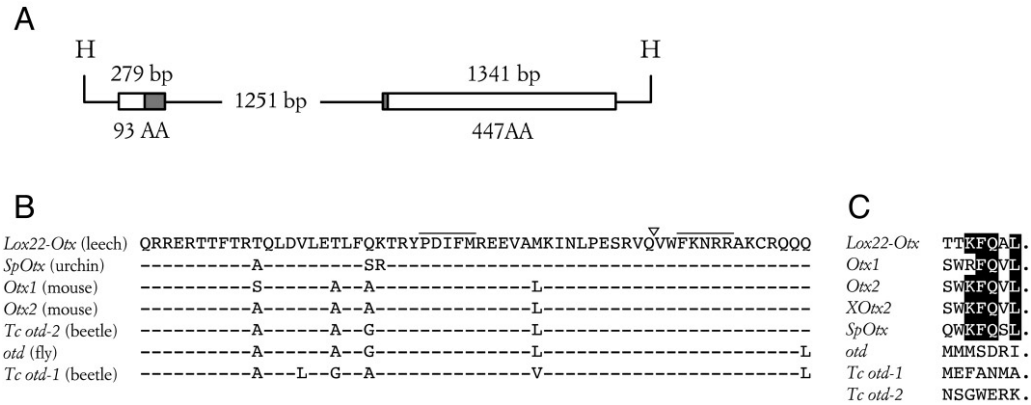

FIG. 1. (A) Schematic diagram of the *Lox22-Otx* genomic clone. Boxes indicate the two long ORFs, with base pair and deduced amino-acid lengths. The hatched region corresponds to the homeobox, which is split by an intron. H, *Hin*dIII restriction sites. (B) Comparison of *Lox22-Otx* homeodomain sequence with Otx genes from other phyla. Identical amino acids are marked by a dash; the inverted triangle marks the location of a conserved intron. Codons used as PCR primers are delineated by horizontal lines. (C) Conservation of amino-acid sequence between the 3′-end of the downstream *Lox22-Otx* ORF and the C-terminal of Otx proteins from other species. Conserved residues are boxed. Sequences are taken from: *SpOtx*—Gan *et al.,* 1995; *Otx1, Otx2*—Simeone *et al.,* 1993; *otd*—Finkelstein *et al.,* 1990; *Tc Otd1, Tc Otd2*—Li *et al.,* 1996; *Xotx2*—Pannese *et al.,* 1995.

ization was performed for 12 h at 65°C in 0.375 M Na_2HPO_4 (pH 7.2), 1 m*M* EDTA (pH 8.0), and 5% SDS with ^{32}P-labeled probe. The radiolabeled probe was generated by PCR following the method of Schowalter and Sommer (1989). Positive clones were identified by autoradiography and purified by secondary screening.

Phage DNA was prepared from a single positive genomic clone by standard techniques using PEG-8000 precipitation and the Promega phage purification kit, followed by treatment with 4 μg/ml RNase A for 25 min at 37°C. Purified phage DNA was digested with a panel of restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon (Amersham), and hybridized with the *Lox22-Otx* homeobox probe as described above. A hybridizing 3.3-kb *Hin*dIII fragment was cloned into Bluescript KS$^+$ (Stratagene) and fully sequenced on both strands.

In Situ Hybridization

The cellular distribution of *Lox22-Otx* message was examined at different stages in development by nonradioactive whole-mount *in situ* hybridization using the method of Nardelli-Haefliger and Shankland (1992), with certain modifications. The initial fixation was lengthened from 30 min to 1 h, and probes were hydrolyzed for 30 min prior to use. For embryos at stage 8 or younger the vitelline membrane was dissected away prior to hybridization, and protease treatment was shortened to 0–10 min. Riboprobes were generated using T7 mMessage mMachine kit (Ambion) without capping. Both sense and antisense probes were obtained by *in vitro* transcription of a 1758-bp fragment of the 3′ exon, including 1341 bp of coding sequence and 417 bp of presumed 3′ UTR. Hybridized tissues were observed in whole mount and following dissection of the nerve cord, using either brightfield or DIC optics.

RESULTS

Isolation and Sequencing of Lox22-Otx

An 86-bp fragment of the *Lox22-Otx* homeobox was isolated from *H. triserialis* stage 9 cDNA by PCR amplification using degenerate oligonucleotide primers specifically designed to target homologues of the *Drosophila* gene *otd* (see Materials and Methods). One cloned PCR fragment encoded a homeodomain sequence very similar to that of *otd* (27 of 28 identical amino acids; see Fig. 1B). This fragment was used to screen an *H. triserialis* genomic library, positive clones were selected, and a 3.3-kb *Hin*dIII restriction fragment containing that homeobox was subcloned and sequenced in full. The gene in question has been designated *Lox22-Otx,* in keeping with the standard nomenclature for leech homeobox genes. The complete sequence of the *Lox22-Otx* genomic fragment has been deposited in the EMBL Library database under Accession No. AF004590. Attempts to isolate the corresponding sequence from a *Helobdella* cDNA library were unsuccessful.

Sequence analysis of the *Lox22-Otx* genomic fragment reveals that the homedomain is interrupted by a 1251-bp intron positioned between codons 46 and 47 (Figs. 1A and 1B). This intron location is conserved among all Otx genes for which genomic organization is known (Simeone *et al.,* 1993; Vandendries *et al.,* 1997), and conceptual splicing of the *Lox22-Otx* homeobox predicts a homeodomain sequence similar to Otx genes from a variety of different species (Fig. 1B). The *Lox22-Otx* homeodomain is most similar (57/60 identical amino acids) to the sea urchin gene *SpOtx* (Gan *et al.,* 1995), and is identical to *otd* at 55/60

amino acids. As with other Otx genes, the *Lox22-Otx* homeodomain has relatively unusual amino acid residue (lysine) at position 50. This amino acid is located in the third recognition helix, which has been shown to be important in determining the DNA-binding specificity of homeodomain proteins (Hanes and Brent, 1989; Treisman *et al.*, 1989).

The fragment of the homeobox that is situated upstream of the intron lies at the 3′ end of a 279-bp open-reading frame (ORF), and the downstream fragment lies at the 5′ end of a 1341-bp ORF. The protein sequence outside the homeobox bears minimal similarity to other reported gene products, with the exception of a short sequence motif at the 3′ end of the downstream ORF. Four of the 5 terminal amino acids in the downstream ORF are identical to the C-terminal residues of Otx gene products reported from mammals, zebrafish, chicken, frog, and sea urchin (Fig. 1C), although a comparable motif is not found in Otx proteins from either of the two insects that have been examined (Finkelstein *et al.*, 1990; Li *et al.*, 1996). Another extrahomeodomain sequence motif is conserved between Otx gene products from vertebrates, sea urchin, and one of two orthologous genes from the beetle *Tribolium* (Li *et al.*, 1996), but this latter sequence is not present in either *Lox22-Otx* or the *Drosophila otd* gene.

The conserved 3′ coding sequence suggests that this downstream ORF represents the C-terminal exon of the *Lox22-Otx* gene. However, the upstream coding sequence is most likely incomplete. There is no start codon (methionine) in the ORF that includes the 5′ end of the homeodomain. Moreover, all Otx genes for which the genomic structure has been established have one or more upstream introns (Simeone *et al.*, 1993; Vandendries *et al.*, 1997), and there is a plausible splice acceptor site (CAGG) located 20 bp upstream of the *Lox22-Otx* homeodomain. Thus, it seems likely that the 3.3-kb *Hin*dIII fragment does not include the complete 5′ coding sequence.

Expression

In order to examine the spatial and temporal expression of *Lox22-Otx* RNA during *Helobdella* embryogenesis, digoxigenin-labeled antisense riboprobes were hybridized to fixed, permeabilized embryos of various developmental stages. Bound probe was visualized by staining with alkaline phosphatase (AP)–antidigoxigenin followed by histochemical reaction. The *Lox22-Otx* hybridization pattern was distinct from that seen with other leech homeobox genes, and sense probes gave either no staining or diffuse staining at all stages. These findings suggest that the hybridization patterns reported here represent the distribution of *Lox22-Otx* RNA. Expression was detected in three principal locations within the prostomial ectoderm which will be described separately.

Surface ectoderm. *Lox22-Otx* expression is first detected near the end of embryonic stage 8. At this time, the spherical embryo consists of a large central yolk mass surrounded by a ribbon-like germinal plate that will later proliferate to form the tissues of the juvenile body wall. The prostomium is located at the extreme anterior end of the germinal plate, and *Lox22-Otx* expression first becomes apparent in a narrow crescent of tissue around the perimeter of this domain (Fig. 2A). The initial hybridization pattern appears to be confined to the epidermal layer of the surface ectoderm, although we cannot rule out the possibility that other closely apposed cells (e.g., peripheral neurons) may be staining as well.

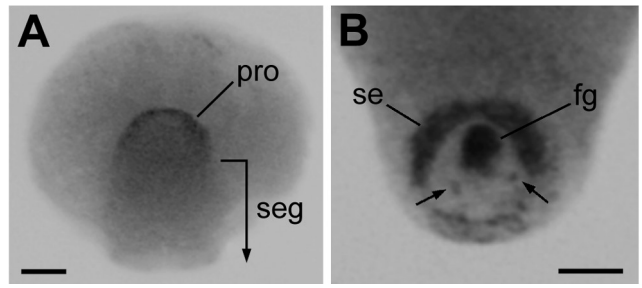

FIG. 2. Early expression of *Lox22-Otx* RNA as revealed by *in situ* hybridization of *Helobdella* embryos. Dorsal is toward the top. (A) Expression is first detected near the end of stage 8 in a crescent of ectodermal tissue encircling the free edges of the prostomium (pr). This unsegmented head region lies in front of the trunk segments (seg) at the future anterior end of the germinal plate. At this stage the germinal plate is a narrow ribbon of tissue wrapped around a central yolk mass. (B) During stage 9 the head end of the germinal plate extends away from the yolk mass, and the domain of *Lox22-Otx* RNA expression expands to form a complete circle of surface ectoderm (se) around the perimeter of the prostomium. Note that hybridization remains more intense on the dorsal side. Intense expression is also observed at the center of the foregut primordium (fg). In the space between the foregut and the ectodermal hybridization, arrows mark two clusters of hybridizing cells that are located in the head ganglion. Scale bars, 50 μm.

Shortly after the onset of embryonic stage 9, the embryo begins to transform into its elongate juvenile morphology, and in doing so the anterior end of the germinal plate pulls away from the central yolk mass. At this time the initial crescent of *Lox22-Otx* expression pattern expands ventrally to completely encircle the prostomium (Figs. 2B and 3), although it remains more intense on the dorsal side (Figs. 2B and 3). However, the ring is wider on its ventral aspect because expression extends from the micromere-derived prostomial ectoderm into the embryonic adhesive gland (Figs. 3A and 3B), a structure that lies between the prostomium and the epidermis of the first body segment (Weisblat *et al.*, 1984). In some cases this ring of hybridizing tissue resolves into separate dorsal and ventral crescents, with little or no staining laterally.

As the embryo progresses into stage 9 a circular groove forms along the inner edge of the ring of *Lox22-Otx* expression (Figs. 3B, 4B, and 4C), and demarcates the foregut primordium—located at the center of the prostomium—

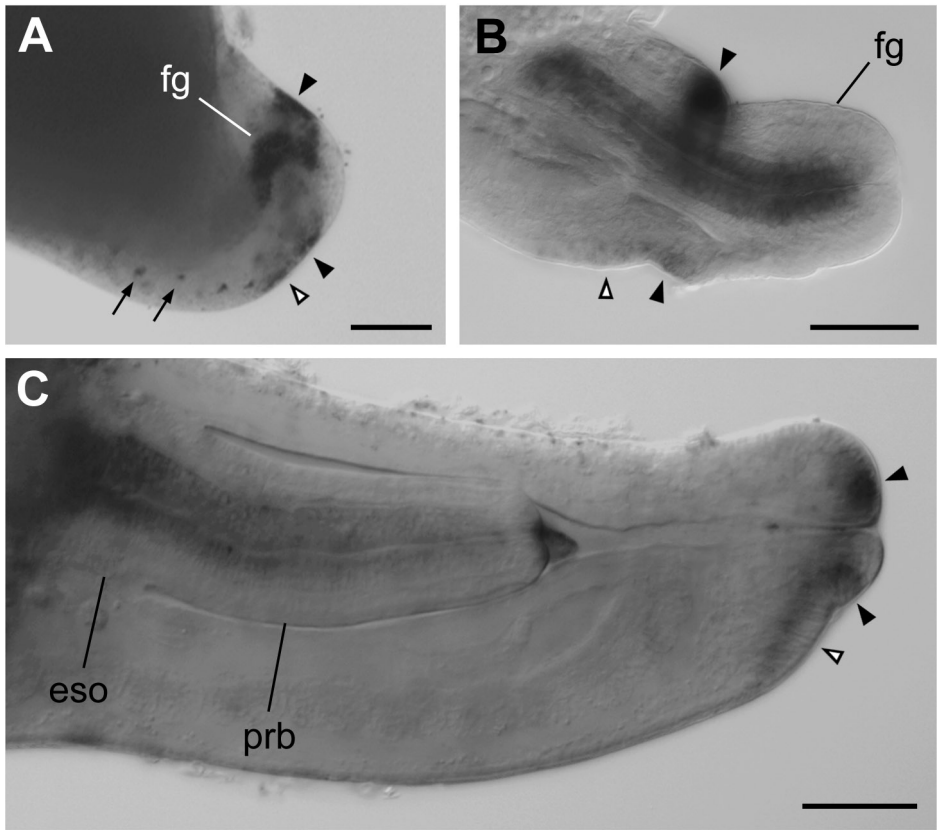

FIG. 3. Expression of *Lox22-Otx* during the latter stages of prostomial morphogenesis as revealed by *in situ* hybridization. Dorsal is toward the top, and anterior to the right. (A) Early in stage 9 RNA expression is observed in a column of foregut tissue (fg) extending from the head domain to the yolk-filled midgut. (Compare with Fig. 4.) Note that the most external portion of the foregut does not show detectable expression, but is encircled by a ring of expression in the surface ectoderm. This ring of expression is here seen from the side, and the black arrowheads mark its dorsal and ventral extent. The white arrowhead marks a contiguous patch of expression in the embryonic adhesive gland, and the arrows point to single neurons expressing this RNA in the more posteriorly located ganglia of the segmental nerve cord. (B) By stage 10 the foregut has grown in length and diameter and protrudes from head domain externally. Within the foregut *Lox22-Otx* expression is restricted to the innermost of two thick tubes of muscle surrounding a central lumen. The foregut protrusion is separated from the surrounding ectoderm by a groove that is deeper on the dorsal side. Black arrowheads mark the dorsal and ventral extent of a ring of *Lox22-Otx* expression in the surrounding ectoderm. Hybridization is also evident in the adhesive gland (white arrowhead), which is separated from the remainder of the ectodermal ring by a second groove. (C) By early stage 11 the foregut has been internalized, and has differentiated into a discrete esophagus (eso) and proboscis (prb), as well as a thin sheath connecting the proboscis to the mouth. In both organs, hybridization is clearly restricted to the innermost of the two thick muscular layers. Internalization of the foregut has pulled the ring of ectodermal expression (black arrowheads) together around the mouth, although there is still a small area of unlabeled ectoderm immediately ventral to the mouth opening. Expression is still evident in the remnant of the embryonic adhesive gland (white arrowhead) as well. The intense "plug" of staining located immediately in front of the proboscis in the foregut lumen is acellular, and presumed to be artifactual. Scale bars, 50 μm.

from the surrounding epithelium. A second groove forms on the ventral side and separates the adhesive gland from the more anterior head tissues (Fig. 3B).

Epidermal expression persists through stage 11, the latest age examined. In *Helobdella* the foregut is withdrawn into the anterior body cavity during the latter part of embryonic stage 10 (see below), and at that time the annular pattern of *Lox22-Otx* epidermal expression is drawn together about the mouth like a pursestring (Fig. 3C).

Foregut. The foregut originates at the center of the prostomium, and eventually differentiates to form the esophagus, the proboscis, the proboscis sheath, and the lining of the oral cavity (Fig. 4). The morphogenesis of the *Helobdella* foregut is complicated, and will be described briefly prior to a discussion of *Lox22-Otx* expression.

By the end of embryonic stage 8, the foregut is a column of dense tissue extending from the surface of the prosto-

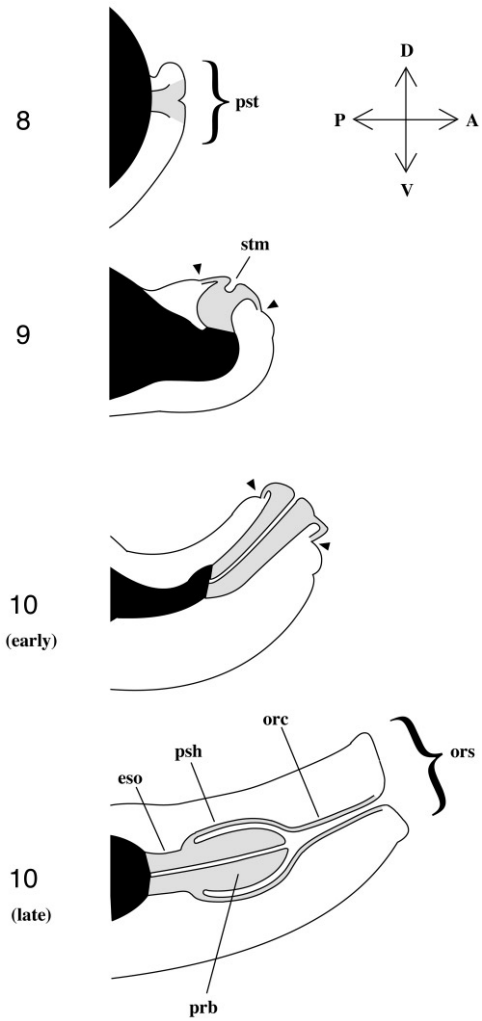

FIG. 4. Foregut morphogenesis of *H. triserialis*, shown in side-view. At embryonic stage 8, the foregut primordium (marked in gray) consists of a column of tissue extending from the center of the prostomial head domain (pro) externally to the yolk-filled midgut primordium (marked in black) internally. During stage 9, the head domain pulls away from the midgut primordium, and the foregut elongates. Note the formation of a thin epithelial layer of foregut tissue connecting the main column to the surface ectoderm, and the appearance of a circular groove (marked by arrowheads) delineating the boundary between ectoderm and foregut. By this stage there is an obvious stomadeal invagination (stm) located at the center of the foregut protrusion. Elongation of the foregut continues during the early part of stage 10, and an obvious lumen comes to extend throughout the column's length. During the latter part of stage 10 the external portion of the foregut is withdrawn into the anterior body cavity, and differentiates to form an extensible proboscis (prb) that is connected to the midgut by a short esophagus (eso). The proboscis is surrounded by a thin proboscis sheath (psh), which is continuous with the lining of the oral cavity (orc). Note that the stomadeum corresponds to the outer end of the proboscis lumen, not to the definitive mouth which is situated near the center of a muscular oral sucker (ors). The proboscis becomes longer and more cylindrical as the embryo matures (see Fig. 3C).

mium externally to the yolk-filled midgut primordium internally (Fig. 4). This initially squat column of tissue elongates as the head pulls away from the yolk mass during stages 9–10, and its outer end becomes a clearly defined prostomial protrusion (Fig. 3B). A central lumen also becomes apparent at this time.

During the latter half of stage 10 the foregut is internalized into the anterior body cavity (Figs. 3C and 4) by a series of rapid and reversible muscular contractions, and this internalized tube differentiates into the proboscis—which is thereafter everted only during feeding—and a short esophagus connecting the proboscis to the midgut (Fig. 3C). Thus, the stomadeal invagination observed at the center of the prostomium at stages 8–9 (Nardelli-Haefliger and Shankland, 1993) becomes the distal end of the proboscis lumen, not the mouth opening of the adult leech. The lining of the oral cavity arises from a thin epithelial layer that is pulled internally with the proboscis, but it is not obvious whether the proboscis sheath also arises from internalized surface ectoderm or delaminates from the proboscis instead (Fig. 4).

Shortly after the onset of epidermal expression—i.e., at the transition from stage 8 to stage 9—intense *Lox22-Otx* expression appears in a thin column of tissue located at the center of the foregut primordium (Figs. 2B and 3A). Expression extends from the midgut yolk mass internally, but does not quite reach the surface of the foregut protrusion externally. As the foregut differentiates, this central column of hybridizing tissue becomes the innermost of the two thick concentric layers of mixed radial and longitudinal muscle that form the proboscis and esophagus (Figs. 3B and 3C). *Lox22-Otx* RNA was detected throughout the complete circumference of the innermost muscle layer, although in some specimens it appeared to be slightly more intense toward the dorsal side. In glossiphoniid leeches these two thick muscle layers are separated by a thin layer of circular muscle (Sawyer, 1986), and in whole-mount preparations we were unable to detect clear expression of *Lox22-Otx* RNA in that intervening layer. There was an obvious decrease in the intensity of foregut staining during stage 11, but this same phenomenon is seen with other riboprobes (Nardelli-Haefliger and Shankland, 1992) and may simply reflect a decreased penetration of probe into deep tissues that are composed of closely packed cells.

During stage 9 and early stage 10, we often saw a much fainter hybridization over the external surface of the foregut protrusion. This latter staining was uniformly distributed around the circumference of the protrusion and seemed to be localized to those tissues that would form the lining of oral cavity following internalization.

In late-stage embryos we consistently observed an irregularly shaped "plug" of hybridization reaction product located in the foregut lumen immediately anterior to the proboscis (Fig. 3C). This staining appears to be associated with acellular material, and is therefore assumed to be a staining artifact.

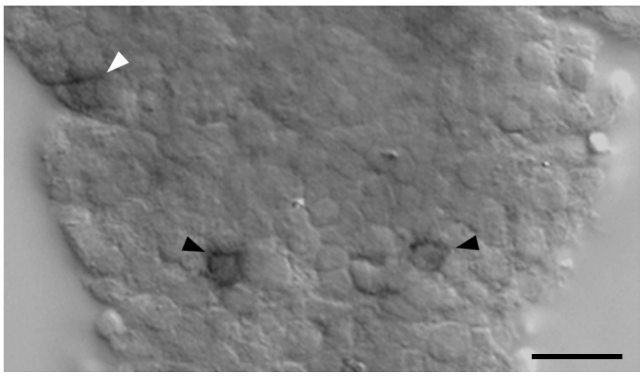

FIG. 5. *Lox22-Otx* RNA is expressed in a small number of neurons in the segmental ganglia of the trunk nervous system, seen here in dissection with anterior toward the top. The fourth rostral neuromere has a bilateral pair of *Lox22-Otx*-expressing neurons (black arrowheads) situated on its ventral surface near the posterior edge. A second, more dorsal pair of hybridizing neurons is out of focus in this ganglion, but one member of this second pair is visible in the next anterior neuromere (white arrowhead). Scale bar, 10 μm.

Central nervous system. Expression of *Lox22-Otx* also appears in the central nervous system (CNS) at the stage 8/9 transition, coincident with the onset of foregut expression (Fig. 2B). At this time the majority of central neurons are undergoing terminal differentiation (Stuart *et al.*, 1987), and based on their size most or all of the hybridizing cell bodies are likely to be postmitotic neurons. In contrast to other aspects of the *Lox22-Otx* expression pattern, neuronal hybridization was observed both in the "supraesophageal" ganglion that encircles the foregut primordium within the unsegmented head domain (Figs. 2B and 6), and also in the segmental neuromeres of the body trunk (Figs. 3A and 5).

In the head ganglion, hybridizing cell bodies were typically observed at several disparate locations around the ganglion's circumference (Fig. 6). The most reliably stained cells included one or two located at the dorsal midline of the ganglion, and three small clusters distributed bilaterally at various locations around the ganglion. The largest of these clusters consists of at least three intensely hybridizing cell bodies, and is located midway along the ganglion's length.

In the body trunk, *Lox22-Otx* expression is restricted to only 1–2 bilateral pairs of neurons/segment (Figs. 5, 6). During stage 9, we consistently observed *Lox22-Otx*-hybridizing neurons in the four fused neuromeres of the subesophageal ganglion, and also in the most anterior midbody ganglia. There was a graded decrease in the intensity of neuronal staining more posteriorly, but in a few specimens we were able to detect *Lox22-Otx*-expressing neurons extending through all 32 segmental neuromeres. The pattern of hybridizing neurons shows some segmental differentiation (Fig. 6), but all segments have what appears to be the same bilateral pair of hybridizing cells situated in the dorsolateral region of the ganglion. In the fourth rostral neuromere and all of the more posterior midbody ganglia and caudal neuromeres, there is a second pair of more ventral neurons that is consistently stained near the ganglion's posterior edge (Figs. 5 and 6).

Extraembryonic tissues. In addition to generating tissues in the prostomium and foregut, the micromeres of the leech embryo also give rise to the epithelial layer of the provisional integument (Smith and Weisblat, 1994), a transient yolk sac that is not thought to contribute to the mature body plan. *Lox22-Otx* probes did not hybridize in the provisional integument during the period of gastrulation. However, when the right and left sides of the germinal plate complete dorsal closure at the end of embryonic stage 10, the last remnants of the provisional integument (presumably undergoing histolysis at this stage) do hybridize to *Lox22-Otx* probes along the seam of dorsal closure. Staining of this structure has also been observed with *Lox22-Otx* sense probes and antisense riboprobes from other genes (unpublished results), and it may reflect a histological artifact rather than *de novo* gene expression.

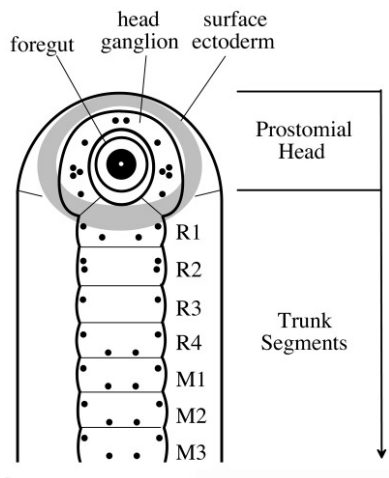

FIG. 6. Schematic representation of *Lox22-Otx* RNA expression in the leech germinal plate around the time of the stage 8/9 transition. The anterior end of the germinal plate is oriented toward the top. *Lox22-Otx* displays a radially organized pattern of expression in the prostomial head domain, including (i) the innermost of two cylinders of tissue comprising the foregut, (ii) single neurons and clusters of neurons (black dots) in the supraesophageal head ganglion that encircles the foregut, and (iii) a concentric annulus of surface ectoderm (shown in gray) that lies near the outer perimeter of the head domain. In addition, *Lox22-Otx* is expressed in 1–2 bilateral pairs of neurons in each segmental ganglion of the trunk nervous system. The exact pattern of expressing neurons varies among the four rostral segments (R1–R4), but is similar in R4 and the midbody ganglia.

DISCUSSION

Molecular Anaysis

Sequence analysis indicates that *Lox22-Otx* is a leech orthologue of the *Drosophila otd* gene and the vertebrate Otx genes. The predicted *Lox22-Otx* homeodomain shares a high level of amino-acid sequence identity with all of the other known genes in this group. This sequence includes a lysine at position 50, a characteristic shared with very few other homeobox genes (Bürglin, 1994). In addition, *Lox22-Otx* is similar to other known Otx genes in that it has an intron located between residues 46 and 47 of the homeodomain coding sequence (Simeone *et al.*, 1993; Vandendries *et al.*, 1997).

Lox22-Otx shares little sequence identity with other known genes outside of the homeodomain and immediately flanking regions. The one noteworthy exception is a short stretch of amino acids at the C-terminus of the predicted *Lox22-Otx* protein. This short sequence is conserved (4/5 amino acids) with the C-terminus of nearly all Otx gene products reported from vertebrates and urchins, but is not apparent in any of three Otx gene products reported from insects. It should be noted that Li *et al.*, (1996) identified a different (and unnamed) peptide sequence located between the homeodomain and the C-terminus that is conserved between vertebrates, urchins, and one of two beetle genes, but this latter sequence is not evident in either the leech or fly genes. It seems likely that both of these extrahomeodomain sequence motifs originated prior to the evolutionary separation of the protostomes and deuterostomes, and that they have independently lost in different subsets of descendant clades or—in clades whose Otx gene has undergone duplication—in only one of two paralogue genes (Li *et al.*, 1996).

Embryonic Expression

The spatial and temporal expression of *Lox22-Otx* RNA was examined by *in situ* hybridization of *Helobdella* embryos at various developmental stages. *Lox22-Otx* transcripts were first detected at the end of embryonic stage 8, a time when the segmental body plan has already been established and organogenesis is beginning in the most anterior—i.e., oldest—portions of the germinal plate. Expression occurs in all of the major ectodermal tissues of the unsegmented head domain, including the epidermis, nervous system, and foregut. In contrast, there is little expression of *Lox22-Otx* RNA in the segmented body trunk, with hybridization being restricted to only 1–2 central neurons/hemisegment.

The expression pattern observed in leeches is in many ways reminiscent of that reported for Otx genes in *Drosophila* and *Tribolium*. These insects show early expression throughout a substantial portion of the head domain (the antennal and preantennal segments—Finkelstein and Perrimon, 1991; Li *et al.*, 1996), and loss-of-function *Drosophila* mutants exhibit serious defects in head development (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). Both insects also show a somewhat later expression of *otd/Otx* in a small number of cells throughout the entire length of the differentiating nervous system. Li *et al.*, (1996) propose that the role of Otx genes in head patterning is primitive, and that the more limited expression in the trunk nervous system reflects a more recent cooptation of the gene.

In *Helobdella*, the cell lineages that generate the head and trunk are segregated during the earliest embryonic cleavages (Shankland and Savage, 1997), and ablation studies indicate that most embryonic blastomeres are differentially specified from an early stage (Blair and Weisblat, 1982; Smith *et al.*, 1996). Detectable expression of *Lox22-Otx* does not commence until several days later in development, and hence it seems unlikely that zygotic expression of this gene plays any role in the initial restriction of the head and trunk lineages. However, the Otx gene may have duplicated (as seen both in vertebrates and the beetle *Tribolium*, Li *et al.*, 1996) during the evolutionary history of the leech, and we cannot at this time rule out the possibility that the leech may have a second Otx orthologue expressed at those earlier stages.

Lox22-Otx would seem a good candidate for defining tissue domains of common morphogenetic potential during later development of the head domain. In the surface ectoderm, *Lox22-Otx* RNA is expressed in an annular pattern whose inner border is largely coincident with the future boundary between the definitive epidermis and the foregut tissues. In the foregut itself, *Lox22-Otx* is expressed in a central core of cells that differentiate to form the innermost of the concentric layers of foregut musclature. Thus, expression of *Lox22-Otx* does not obviously correlate with any particular pathway of cytodifferentiation, but rather with the subdivision of these two organ systems into distinct morphogenetic zones. On the other hand, expression of *Lox22-Otx* in the CNS involves fewer cells and is more dispersed, suggesting that it is involved in the development of only a small subset of neuronal phenotypes.

Conservation of the Bilaterian Body Plan

As schematized in Fig. 7, the findings presented here confirm and extend the idea that multiple bilaterian phyla share a common ancestral body plan in which the head and trunk regions of the AP axis are genetically distinct (Holland, 1992; Arendt and Nübler-Jung, 1996). Otx genes are expressed predominately in the extreme anterior region of various chordates (Finkelstein and Perrimon, 1990; Williams and Holland, 1996; Wada *et al.*, 1996), two species of insect (Finkelstein and Perrimon, 1990; Li *et al.*, 1996), and the leech *Helobdella*. Moreover, this gene has been shown by mutation to be necessary for development of large regions of head tissue in both *Drosophila* (Finkelstein and Perrimon, 1990; Cohen and Jürgens, 1990) and mice (Acampora *et al.*, 1995, 1996). The Otx gene shows a variable and not obviously homologous expression pattern among echi-

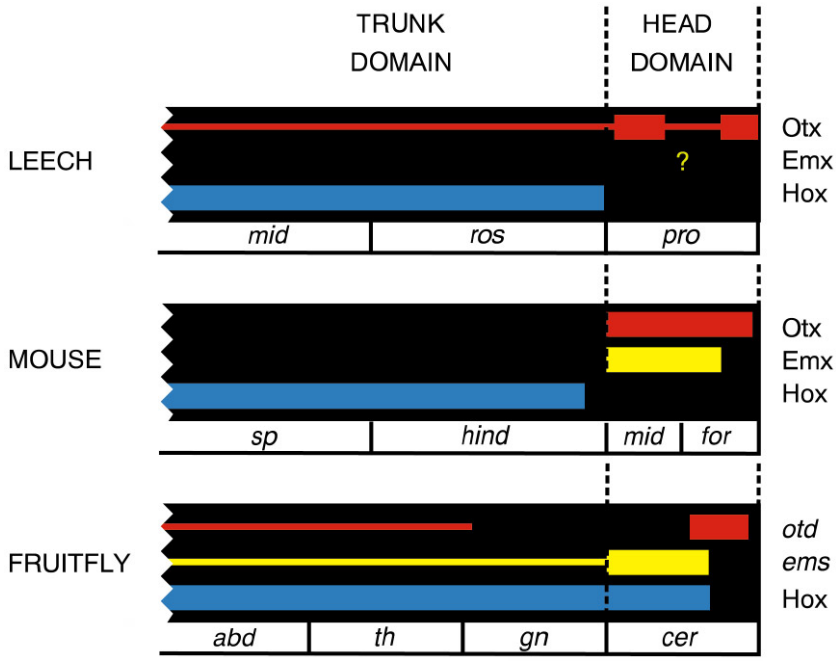

FIG. 7. Comparison of gene expression domains that appear to define an evolutionarily conserved head/trunk distinction in the leech, mouse, and fruitfly. Anterior is to the right. Thick bars show AP domains of widespread expression. Thin bars show regions in which a gene is expressed in only a few differentiating cell types, and does not appear to be responsible for early pattern formation. Blue bars show the cumulative domain of expression for the entire Hox gene cluster(s), and in mouse the red and yellow bars show cumulative data for *Otx1/Otx2* and *Emx1/Emx2* genes, respectively. Mouse and fruitfly expression domains are taken from Thor (1995). Leech Hox gene expression domain is taken from Kourakis *et al.*, (1997). Head expression of leech *Otx* is portrayed as bimodal to reflect the separate zones of expression at the center of the foregut and near the outer rim of the surface ectoderm. Leech: *mid,* midbody segments; *ros,* rostral segments; *pro,* prostomium. Mouse: *fore,* forebrain; *mid,* midbrain; *hind,* hindbrain rhombomeres; *sp,* spinal segments. Fruitfly: *cer,* cerebral segments; *gn,* gnathal segments; *tr,* thorax; *abd,* abdomen.

noderms (Lowe and Wray, 1997), but this discrepancy is very likely associated with the fact that the adult echinoderm body plan has been extensively modified from that of other Bilateria (Brusca and Brusca, 1990). Further evidence of a genetically defined head domain conserved among other more typical bilaterians has come from interphyletic comparison of developmental regulatory genes such as the Emx homeobox gene and the nuclear steroid receptor gene *tailless* (Thor, 1995).

Additional support for a conserved genetic distinction between the head and trunk regions can be found in the fact that Hox gene expression—responsible for patterning most of the AP axis (McGinnis and Krumlauf, 1992)—is largely excluded from the genetically defined head domain in many Bilatera (Fig. 7). In vertebrates such as the mouse, Hox gene expression is restricted to the hindbrain rhombomeres and yet more posterior spinal segments, whereas genes like Otx and Emx are only expressed more anteriorly in the forebrain. In the leech as well, none of the known Hox genes (including a *lab* orthologue) are expressed within the prostomial head domain (Kourakis *et al.*, 1997), the predominant site of *Lox22-Otx* expression. Some degree of overlap is observed in the *Drosophila* embryo, where the expression domain of the most anterior Hox gene (*lab*) is coincident with the expression of *ems,* although still posterior to the domain of *otd* expression (Fig. 7). But in *Drosophila* there is yet further evidence of genetic distinctions between head and trunk, since the Pair Rule genes that establish the number and periodicity of the 14 trunk parasegments do not play this same role in the head domain, where genes such as *otd* and *ems* are required instead for normal segment formation (Cohen and Jürgens, 1991).

It should be noted that "head" and "trunk" as defined here by genetic pathways do not necessarily correspond to the common anatomical use of these terms for a given taxon. For instance, the insect head is defined on anatomical grounds as including both the four cephalic segments that are patterned by head genes (Schmidt-Ott and Technau, 1992) and the next three gnathal (i.e., mouthpart-bearing) segments that express Pair Rule and Hox genes like the thoracic and abdominal segments behind them (Finkelstein and Perrimon, 1991). The gnathal appendages of modern insects function exclusively as mouthparts, but it is generally held that they evolved from limbs morphologically similar to those found on more posterior segments in primitive arthropods (Manton, 1977). A similar phenom-

enon can be found in the vertebrate head, which is thought to have evolved by a novel integration of tissues derived from cephalic neural crest and hindbrain—e.g., the most anterior sites of Hox gene expression (McGinnis and Krumlauf, 1992)—and tissues derived from yet more anterior structures (Gans and Northcutt, 1983). One can speculate that the border between the genetically defined head and trunk domains of modern Bilateria was in fact a significant anatomical boundary in an early bilaterian ancestor. But even if this were the case, the anatomical manifestation of that boundary has been obscured in at least some descendant lineages by adaptations that functionally integrated tissues from both sides of the boundary.

It is also worth noting that two of the downstream genes in the *Drosophila* segmentation pathway—*en* and *wingless (wg)*—are expressed in a similar manner in both trunk and head segments (Schmidt-Ott and Technau, 1992). This is not the case in leech *Helobdella,* whose *ht-en* gene is expressed in all 32 trunk segments but not at all in the prostomial head (Wedeen and Weisblat, 1991). If one assumes that an unsegmented head domain represents the primitive bilaterian condition, then insects such as *Drosophila* may have coopted *en* and *wg* expression into their head domain as a developmental mechanism for generating preoral head segments. Preoral segments are a characteristic of many arthropod groups (Manton, 1977), and it will be of interest to learn where the genetically defined head/trunk boundary is situated in the body plan of other arthropods.

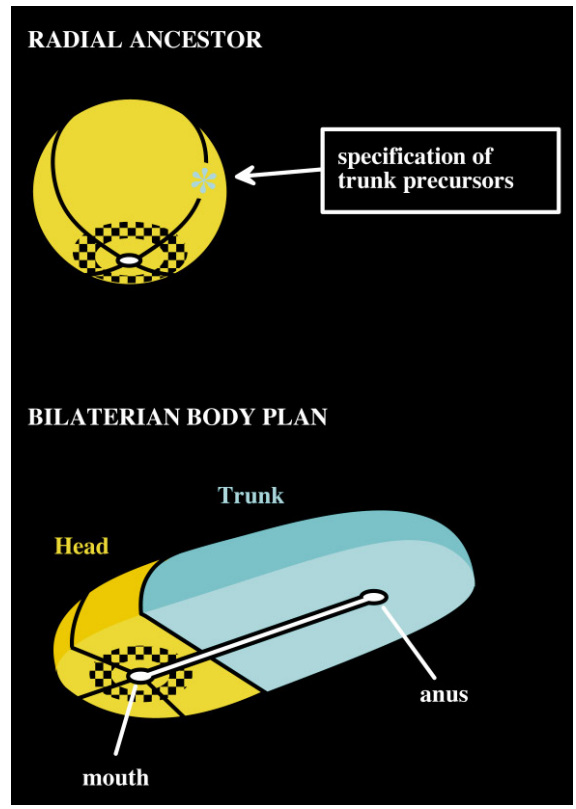

FIG. 8. Model for the evolutionary origin of the bilaterian body plan from a radially organized ancestor. It is here assumed that the bilaterian head domain arose by a remodeling of the ancestral body plan, thus accounting for the radial organization of gene expression patterns and ectodermal cell lineages (Nardelli-Haefliger and Shankland, 1993) in the leech prostomium. The bilaterian trunk domain is portrayed as arising by specification—and subsequent allometric expansion—of a cell population restricted to one side of the radial ancestor. Certain of the genes involved in patterning the ancestral body plan became restricted to the head domain of Bilateria as a consequence, and one such putative expression domain is here characterized by a ring of shaded tissues. The mouth and anus of the bilaterian digestive tract are portrayed as arising from opposite ends of a "slit" blastopore, as is common among spiralian protostomes.

Origin of the Bilaterian Body Plan

How did the bilaterian body plan originate? There is a growing consensus from molecular phylogenies that the Bilateria are a monophyletic clade whose closest relatives are the radially organized cnidarians and ctenophores (Raff, 1996). However, there is still room for uncertainty, and other phylogenies have been proposed (Nielsen, 1995). Our present findings reveal that even though the leech has a typical bilaterian body plan, it also displays a marked tendency toward radial organization within the prostomial head domain. The pattern of *Lox22-Otx* expression is largely concentric about the mouth, with relatively minor variations from radial symmetry. This trend toward radial organization is also mirrored by the deployment of the prostomial cell lineages. During the formation of the leech's germinal plate, the four primary micromere clones that comprise the prostomial ectoderm are arranged as radial wedges about the mouth (Nardelli-Haefliger and Shankland, 1993), and there is a strikingly similar quadriradial organization of cell lineages in the larvae of other, more basal annelids as well (see discussion in Shankland, 1998).

If in fact bilaterian animals did evolve from a radial ancestor—as has been suggested by many authors (Brusca and Brusca, 1990; Raff, 1996)—then the radial features of the leech's head domain might reflect the persistence of ancestral patterning mechanisms that preceded the evolution of the bilaterian body plan. One possible scenario is that the Bilateria evolved from a radial ancestor by the addition and subsequent expansion of a genetically distinct anatomical domain that eventually became the bilaterian trunk (Fig. 8). In this model the Otx gene would have originally been involved in the development of a radial ancestor (e.g., specifying cell fates along the oral–aboral axis), and was simply not coopted into the presumably novel genetic pathways for the formation and patterning of the trunk. If this model is correct, the bilaterian "head genes" were in fact relegated to the head domain as a consequence of their evolutionary history.

If the radial characteristics of a prebilaterian ancestor were carried over into the head domain of primitive bilaterians, then an underlying radial organization of the head should also be detectable in other bilaterians. There are currently no data on the expression of head genes in any annelids—or other spiralian protostomes—besides *Helobdella*. Radially organized expression domains have not been described as such in the heads of insects or chordates, but this may be a matter of viewpoint. For instance, the *otd* and *ems* expression patterns seen in *Drosophila* are commonly portrayed as transverse bands marking specific AP positions in the embryonic fate map (Finkelstein and Perrimon, 1991), but in topological terms they are also equivalent to concentric rings about the mouth. Furthermore, one must consider the possibility that ancestral patterns of gene expression could have been obscured in some evolutionary lineages by the imposition of novel axial properties resulting from the functional integration of trunk and head. Indeed, one important feature of our radial head model is the fact that we view the AP axis of bilaterian animals as primarily an attribute of the more recently evolved trunk domain. If this is the case, then AP differentiation would have been imposed onto the bilaterian head secondarily as a result of its anatomical relationship to and functional integration with the trunk.

Consistent with the model presented in Fig. 8, trunk tissues are generated by an anisotropically distributed cell population in the embryos of many extant bilaterians. Spiralians such as the leech originate as four otherwise similar embryonic quadrants, but only one quadrant becomes specialized to generate the stem cells that will produce the mesoderm and ectoderm of the adult trunk (Shankland and Savage, 1997; Shankland, 1998). In chordates, the dorsal side of the blastopore is defined by an "axial" mesoderm whose convergent extension is the major driving force for trunk elongation (Keller *et al.*, 1985). We envision that the bilaterian trunk originated with the appearance of developmentally specialized cells at one meridion of a radial ancestor, and that the trunk was elaborated and expanded during subsequent evolution to become a major or even predominant portion of the adult body plan. This model is consistent with some of the ideas put forward by Davidson *et al.*, (1995), who propose that a central step in the evolution of bilaterian animals was the addition of novel structural domains by specialized cell populations that became "set-aside" during early embryogenesis. However, our model differs in that we do not envision this specialized cell population as replacing the ancestral body plan during the later stages of development, but rather envision it as generating a new anatomical domain that became structurally integrated with the preexisting body plan and eventually caused the latter to become reorganized around a novel set of developmental axes.

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REFERENCES

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., and Brûlet, P. (1995). Forebrain and midbrain regions are deleted in $Otx2^{-/-}$ mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121,** 3279–3290.

Acampora, D., Mazan, S., Avantaggiato, V., Barone, P., Tuorto, F., Lallemand, Y., Brûlet, P., and Simeone, A. (1996). Epilepsy and brain abnormalities in mice lacking the *Otx1* gene. *Nat. Genet.* **14,** 218–222.

Arendt, D., and Nübler-Jung, K. (1996). Common ground plans in early brain development in mice and flies. *BioEssays* **18,** 255–259.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992). "Current Protocols in Molecular Biology." Wiley, New York.

Blair, S. S., and Weisblat, D. A. (1982). Ectodermal interactions during neurogenesis in the glossiphoniid leech *Helobdella triserialis*. *Dev. Biol.* **91,** 64–72.

Boncinelli, E., Gulisano, M., and Broccoli, V. (1993). Emx and Otx homeobox genes in the developing mouse brain. *J. Neurobiol.* **24,** 1356–1366.

Brusca, R. C., and Brusca, G. J. (1990). "Invertebrates." Sinauer, Sunderland, MA.

Bürglin, T. R. (1994). A comprehensive classification of homeobox genes. *In* "Guidebook to the Homeobox Genes" (D. Duboule, Ed.), pp. 25–74. Oxford Univ. Press, New York.

Cohen, S., and Jürgens, G. (1991). *Drosophila* headlines. *Trends Genet.* **7,** 267–272.

Cohen, S. M., and Jürgens, G. (1990). Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature* **346,** 482–488.

Davidson, E. H., Peterson, K. J., and Cameron, R. A. (1995). Origin of bilaterian body plans: Evolution of developmental regulatory mechanisms. *Science* **270,** 1319–1325.

Finkelstein, R., and Perrimon, N. (1990). The *orthodenticle* gene is regulated by *bicoid* and *torso* and specifies *Drosophila* head development. *Nature* **346,** 485–488.

Finkelstein, R., and Perrimon, N. (1991). The molecular genetics of head development in *Drosophila melanogaster*. *Development* **112,** 899–912.

Finkelstein, R., Smouse, D., Capaci, T. M., Spradling, A. C., and Perrimon, N. (1990). The *orthodenticle* gene encodes a novel homeo domain protein involved in the development of the *Drosophila* nervous system and ocellar visual structures. *Genes Dev.* **4,** 1516–1527.

Gan, L., Mao, C.-A., Wikramanayake, A., Angerer, L. M., Angerer, R. C., and Klein, W. H. (1995). An orthodenticle-related protein from *Strongylocentrotus purpuratus*. *Dev. Biol.* **167,** 517–528.

Gans, C., and Northcutt, R. G. (1983). Neural crest and the origin of vertebrates: A new head. *Science* **220,** 268–274.

Hanes, S. D., and Brent, R. (1989). DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell* **57,** 1275–1283.

Holland, P. (1992). Homeobox genes in vertebrate evolution. *BioEssays* **14,** 267–273.

Keller, R. E., Danilchik, M., Gimlich, R., and Shih, J. (1985). The function and mechanism of convergent extension during gastrulation of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **89**(Suppl.), 185–209.

Kourakis, M. J., Master, V. A., Lokhorst, D. K., Nardelli-Haefliger, D., Wedeen, C. J., Martindale, M. Q., and Shankland, M. (1997). Conserved anterior boundaries of Hox gene expression in the central nervous system of the leech *Helobdella*. *Dev. Biol.* **190,** 284–300.

Lawrence, P. A. (1992). "The Making of a Fly." Blackwell Scientific, Oxford.

Li, Y., Allende, M. L., Finkelstein, R., and Weinberg, E. S. (1994). Expression of two zebrafish *orthodenticle*-related genes in the embryonic brain. *Mech. Dev.* **48,** 229–244.

Li, Y., Brown, S. J., Hausdorf, B., Tautz, D., Denell, R. E., and Finkelstein, R. (1996). Two *orthodenticle*-related genes in the short-germ beetle *Tribolium castaneum*. *Dev. Genes Evol.* **206,** 35–45.

Lowe, C. J., and Wray, G. A. (1997). Radical alterations in the roles of homeobox genes during echinoderm evolution. *Nature* **389,** 718–721.

Manton, S. M. (1977). "The Arthropoda: Habits, Functional Morphology and Evolution." Clarendon, Oxford.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68,** 283–302.

Nardelli-Haefliger, D., and Shankland, M. (1992). *Lox2*, a putative leech segment identity gene, is expressed in the same segmental domain in different stem cell lineages. *Development* **116,** 697–710.

Nardelli-Haefliger, D., and Shankland, M. (1993). *Lox10*, a member of the *NK-2* homeobox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech *Helobdella*. *Development* **118,** 877–892.

Nielsen, C. (1995). "Animal Evolution: Interrelationships of the Living Phyla." Oxford Univ. Press, Oxford.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121,** 707–720.

Raff, R. A. (1996). "The Shape of Life." Univ. of Chicago Press, Chicago, IL.

Salser, S. J., Loer, C. M., and Kenyon, C. (1993). Multiple HOM-C gene interactions specify cell fates in the nematode central nervous system. *Genes Dev.* **7,** 1714–1724.

Sawyer, R. T. (1986). "Leech Biology and Behaviour." Clarendon Press, Oxford.

Schmidt-Ott, U., and Technau, G. M. (1992). Expression of *en* and *wg* in the embryonic head and brain of *Drosophila* indictes a refolded band of seven segment remnants. *Development* **116,** 111–125.

Schowalter, D. B., and Sommer, S. S. (1989). The generation of radiolabeled DNA and RNA probes with polymerase chain reaction. *Anal. Biochem.* **177,** 90–94.

Shankland, M. (1998) Anteroposterior pattern formation in the leech embryo. *In* "Cell Lineage and Fate Determination" (S. A. Moody, Ed.), Academic Press, New York.

Shankland, M., and Savage, R. M. (1997). Annelids, the segmented worms. *In* "Embryology: Constructing the Organism" (S. F. Gilbert and A. M. Raunio, Eds.), pp. 219–235. Sinauer Associates, Sunderland, MA.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M. R., Nigro, V., and Boncinelli, E. (1993). A vertebrate gene related to *orthodenticle* contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* **12,** 2735–2747.

Smith, C. M., Lans, D., and Weisblat, D. A. (1996). Cellular mechanisms of epiboly in leech embryos. *Development* **122,** 1885–1894.

Smith, C. M., and Weisblat, D. A. (1994). Micromere fate maps in leech embryos: Lineage-specific differences in rates of cell proliferation. *Development* **120,** 3427–3438.

Stuart, D. K., Blair, S. S., and Weisblat, D. A. (1987). Cell lineage, cell death and the developmental origin of identified serotonin- and dopamine-containing neurons in the leech. *J. Neurosci.* **7,** 1107–1122.

Thor, S. (1995). The genetics of brain development: conserved programs in flies and mice. *Neuron* **15,** 975–977.

Torrence, S. A., and Stuart, D. K. (1986). Gangliogenesis in leech embryos: migration of neural precursor cells. *J. Neurosci.* **6,** 2736–2746.

Treisman, J., Gönczy, P., Vashishtha, M., Harris, E., and Desplan, C. (1989). A single amino acid can determine the DNA binding specificity of homeodomain proteins. *Cell* **59,** 553–562.

Vandendries, E. R., Johnson, D., and Reinke, R. (1997). *orthodenticle* is required for photoreceptor cell development in the *Drosophila* eye. *Dev. Biol.* **173,** 243–255.

Wada, S., Katsuyama, Y., Sato, Y., Ito, C., and Saiga, H. (1996). *Hroth*, an orthodenticle-related homeobox gene of the ascidian, *Halocynthia roretzi:* Its expression and putative roles in the axis formation during embryogenesis. *Mech. Dev.* **60,** 59–71.

Wedeen, C. J., Price, D. J., and Weisblat, D. A. (1990). Analysis of the life cycle, genome, and homeo box genes of the leech *Helobdella triserialis*. *In* "Cellular and Molecular Biology of Pattern Formation" (D. L. Stocum and T. Karr, Eds.), pp. 145–167. Oxford Univ. Press, New York.

Wedeen, C. J., and Weisblat, D. A. (1991). Segmental expression of an *engrailed*-class gene during early development and neurogenesis in an annelid. *Development* **113,** 805–814.

Weisblat, D. A., Harper, G., Stent, G. S., and Sawyer, R. T. (1980). Embryonic cell lineages in the nervous system of the glossiphoniid leech *Helobdella triserialis*. *Dev. Biol.* **76,** 58–78.

Weisblat, D. A., Kim, S. Y., and Stent, G. S. (1984). Embryonic origins of cells in the leech *Helobdella triserialis*. *Dev. Biol.* **104,** 65–85.

Williams, N. A., and Holland, P. W. H. (1996). Old head on young shoulders. *Nature* **383,** 490.

Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S., and Aizawa, S. (1997). *Emx1* and *Emx2* functions in development of dorsal telencephalon. *Development* **124,** 101–111.

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